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PTO/SB/05 (08-00)
Approved for use through 10/31/2002. OMB 0651-0032
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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	1079-2
First Inventor	Kurt Haas et al.
Title	SINGLE CELL ELECTROPORATION
Express Mail Label No.	EL393561383US

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

1. [X] Fee Transmittal Form (e.g., PTO/SB/17) *(Submit an original and a duplicate for fee processing)*
2. [X] Applicant claims small entity status. See 37 CFR 1.27.
3. [X] Specification [*Total Pages* 23] *(preferred arrangement set forth below)*
   - Descriptive title of the invention
   - Cross Reference to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to sequence listing, a table, or a computer program listing appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
4. [X] Drawing(s) *(35 U.S.C. 113)* [*Total Sheets* 4]
5. Oath or Declaration [*Total Pages* 6]
   a. [X] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 CFR 1.63 (d)) *(for continuation/divisional with Box 17 completed)*
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
6. [ ] Application Data Sheet. See 37 CFR 1.76
7. [ ] CD-ROM or CD-R in duplicate, large table or Computer Program *(Appendix)*
8. Nucleotide and/or Amino Acid Sequence Submission *(if applicable, all necessary)*
   a. [ ] Computer Readable Form (CRF)
   b. Specification Sequence Listing on:
      i. [ ] CD-ROM or CD-R (2 copies); or
      ii. [ ] paper
   c. Statements verifying identity of above copies

ADDRESS TO:

Assistant Commissioner for Patents
Box Patent Application
Washington, DC 20231

ACCOMPANYING APPLICATION PARTS

9. [X] Assignment Papers (cover sheet & document(s))
10. [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [X] Power of Attorney
11. [ ] English Translation Document *(if applicable)*
12. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
13. [ ] Preliminary Amendment
14. [X] Return Receipt Postcard (MPEP 503) *(Should be specifically itemized)*
15. [ ] Certified Copy of Priority Document(s) *(if foreign priority is claimed)*
16. [ ] Other: .....

17. If a CONTINUING APPLICATION, *check appropriate box, and supply the requisite information below and in a preliminary amendment, or in an Application Data Sheet under 37 CFR 1.76:*

[ ] Continuation [ ] Divisional [ ] Continuation-in-part (CIP) of prior application No.: _____/_____

Prior application information: *Examiner* _____ *Group / Art Unit:* _____

For CONTINUATION OR DIVISIONAL APPS only: The entire disclosure of the prior application, from which an oath or declaration is supplied under Box 5b, is considered a part of the disclosure of the accompanying continuation or divisional application and is hereby incorporated by reference. The incorporation can only be relied upon when a portion has been inadvertently omitted from the submitted application parts.

18. CORRESPONDENCE ADDRESS

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Name (Print/Type)	Jeffrey S. Steen	*Registration No. (Attorney/Agent)*	32,063
Signature		*Date*	10/10/00

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence and the documents referred to as enclosed are being deposited with the United States Postal Service on date below in an envelope as "Express Mail Post Office to Addressee" Mail Label Number EL393561383US addressed to: Assistant Commissioner for Patents, Box Patent Application, Washington, DC. 20231.

Dated: October 10, 2000

Tidge Holmberg

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FEE TRANSMITTAL for FY 2000

Patent fees are subject to annual revision.

TOTAL AMOUNT OF PAYMENT ($) 486.00

Complete if Known	
Application Number	
Filing Date	
First Named Inventor	Kurt Haas et al.
Examiner Name	
Group Art Unit	
Attorney Docket No.	1079-2

METHOD OF PAYMENT (check one)

1. [X] The Commissioner is hereby authorized to charge indicated fees and credit any overpayments to:
   - Deposit Account Number: 04-1121
   - Deposit Account Name: DILWORTH & BARRESE, LLP
   - [X] Charge Any Additional Fee Required Under 37 CFR 1.16 and 1.17
   - [ ] Applicant claims small entity status. See 37 CFR 1.27
2. [X] **Payment Enclosed:** [X] Check [ ] Credit card [ ] Money Order [ ] Other

FEE CALCULATION

1. BASIC FILING FEE

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
101	710	201	355	Utility filing fee	$355.00
106	320	206	160	Design filing fee	
107	490	207	245	Plant filing fee	
108	710	208	355	Reissue filing fee	
114	150	214	75	Provisional filing fee	

SUBTOTAL (1) ($) 355.00

2. EXTRA CLAIM FEES

		Extra Claims	Fee from below	Fee Paid
Total Claims	29 -20** =	9	x $9	= $81
Independent Claims	2 - 3** =	0	x $40	= 0
Multiple Dependent			$135	= 0

***or number previously paid, if greater; For Reissues, see below*

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description
103	18	203	9	Claims in excess of 20
102	80	202	40	Independent claims in excess of 3
104	270	204	135	Multiple dependent claim, if not paid
109	80	209	40	** Reissue independent claims over original patent
110	18	210	9	** Reissue claims in excess of 20 and over original patent

SUBTOTAL (2) ($) 81.00

FEE CALCULATION (continued)

3. ADDITIONAL FEES

Large Entity Fee Code	Large Entity Fee ($)	Small Entity Fee Code	Small Entity Fee ($)	Fee Description	Fee Paid
105	130	205	65	Surcharge - late filing fee or oath	
127	50	227	25	Surcharge - late provisional filing fee or cover sheet	
139	130	139	130	Non-English specification	
147	2,520	147	2,520	For filing a request for *ex parte* reexamination	
112	920*	112	920*	Requesting publication of SIR prior to Examiner action	
113	1,840*	113	1,840*	Requesting publication of SIR after Examiner action	
115	110	215	55	Extension for reply within first month	
116	390	216	195	Extension for reply within second month	
117	890	217	445	Extension for reply within third month	
118	1,390	218	695	Extension for reply within fourth month	
128	1,890	228	945	Extension for reply within fifth month	
119	310	219	155	Notice of Appeal	
120	310	220	155	Filing a brief in support of an appeal	
121	270	221	135	Request for oral hearing	
138	1,510	138	1,510	Petition to institute a public use proceeding	
140	110	240	55	Petition to revive - unavoidable	
141	1,240	241	620	Petition to revive - unintentional	
142	1,240	242	620	Utility issue fee (or reissue)	
143	440	243	220	Design issue fee	
144	600	244	300	Plant issue fee	
122	130	122	130	Petitions to the Commissioner	
123	50	123	50	Petitions related to provisional applications	
126	240	126	240	Submission of Information Disclosure Stmt	
581	40	581	40	Recording each patent assignment per property (times number of properties)	$40
146	710	246	355	Filing a submission after final rejection (37 CFR § 1.129(a))	
149	710	249	355	For each additional invention to be examined (37 CFR § 1.129(b))	
179	710	279	355	Request for Continued Examination (RCE)	
169	900	169	900	Request for expedited examination of a design application	

Other fee (specify) _____

* Reduced by Basic Filing Fee Paid

SUBTOTAL (3) ($) 40.00

SUBMITTED BY				Complete *(if applicable)*	
Name (Print/Type)	Jeffrey S. Steen	*Registration No. (Attorney/Agent)*	32,063	*Telephone*	(516) 228-8484
Signature				*Date*	10/10/00

CERTIFICATION UNDER 37 C.F.R. § 1.10

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Dated: October 10, 2000

Tidge Holmberg

PATENT
Attorney's Docket No. 1079-2

Applicant or Patentee: Kurt Haas et al.
Serial or Patent No.: 0 /
Filed or Issued:
For: SINGLE CELL ELECTROPORATION

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) and 1.27(d)) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION Cold Spring Harbor Laboratory

ADDRESS OF ORGANIZATION P.O. Box 100, Cold Spring Harbor, NY 11724

TYPE OF ORGANIZATION

☐ University or Other Institution of Higher Education

■ Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3))

☐ Nonprofit Scientific or Educational Under Statute of State of the United States of America

(Name of State ______)

(Citation of Statute ______)

☐ Would Qualify as Tax Exempt Under Internal Revenue Service Code (26 USC 501(a) and 501(c)(3)), if Located in the United States of America

☐ Would Qualify as Nonprofit Scientific or Educational Under Statute of State of the United States of America if Located in the United States of America

(Name of State ______)

(Citation of Statute ______)

I hereby declare that the nonprofit organization defined above qualifies as a nonprofit organization, as defined in 37 CFR 1.9(e), for purposes of paying reduced fees under Sections 41(a) and (b) of Title 35, United States Code, with regard to the invention entitled SINGLE CELL ELECTROPORATION

by inventor(s) Kurt Haas et al.

described in

■ the specification filed herewith.

☐ application serial no. 0 /____, filed ______.

☐ patent no. ______, issued ______.

I hereby declare that rights under contract or law have been conveyed to, and remain with, the nonprofit organization, with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed below* and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 CFR 1.9(d), or by any concern that would not qualify as a small business concern under 37 CFR 1.9(d), or a nonprofit organization under 37 CFR 1.9(e).

**NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27).*

NAME Cold Spring Harbor Laboratory

ADDRESS P.O. Box 100

Cold Spring Harbor, NY 11724

☐ Individual ☐ Small Business Concern ■ Nonprofit Organization

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small business entity is no longer appropriate. (37 CFR 1.28(b).

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING G. Morgan Browne
TITLE IN ORGANIZATION Administrative Director
ADDRESS OF PERSON SIGNING Cold Spring Harbor Laboratory
P.O. Box 100, Cold Spring Harbor, NY 11724

SIGNATURE ______ Date 9/28/00

Docket: 1079-2

SINGLE CELL ELECTROPORATION

GOVERNMENT RIGHTS

This invention was funded, at least in part, under grants from the National Eye Institute of the National Institutes of Health, Nos. EY11261 and EY06922-03. The Government may therefore have certain rights in the invention.

BACKGROUND

Recent large-scale genetic screens have uncovered a multitude of genes implicated in brain development, learning and memory, regeneration, and neurological diseases. Determining the function of these genes in vivo necessitates advanced techniques for controlling the timing and location of gene expression, combined with specific assays of brain cell function or morphology. In some cases it will be important to introduce genes into postmitotic cells which are refractory to most available gene transfer methods. In other cases, introduction of genes of interest into pluripotent cells such as stem cells will be required to determine their function or to provide therapeutic value.

Morphologic studies of neuronal development in Xenopus following viral gene transfer and in transgenic Drosophila exemplify the potential for elucidating gene function when the genetic makeup of individual neurons can be manipulated and the consequences of the gene transfer on neuronal structure can be observed by in vivo imaging techniques (Li, et al., Nature Neurosci *3*, 217-225 (2000), Nedivi, et al., Science *281*, 1863-1866 (1998), Wu G-Y and Cline HT, Science *279,* 222-226 (1998); Zou D-J and Cline HT, Neuron *16*; 529-539 (1996), Davis, et al., Neuron *19*, 561-73 (1997)). As another example, side-by-side comparison of the synaptic properties of individual genetically modified neurons with unaffected neurons in the same brain provides a powerful tool for the elucidation of gene function (Hayashi, et al., Science *287*, 2262-2267 (2000). However, current techniques for spatially and temporally controlled introduction of genes of interest into single cells within central nervous system tissue is limited. One versatile technique applicable to this problem is electroporation.

Electroporation is a popular technique for introducing macromolecules, including DNA, RNA, dyes, proteins and various chemical agents, into cells. Electroporation refers to the permeabilization of cell membranes by application of short duration electric field pulses, traditionally between relatively large plate electrodes (Neumann, et al., Bioelectrochem Bioenerg *48*, 3-16 (1999); Ho, et al., Crit Rev Biotechnol *16*, 349-62 (1996)). Large external electric fields induce high transmembrane potentials leading to the formation of minute pores (20-120 nm diameter) restricted to small regions of the cell membrane (< 0.1%) adjacent to the electrodes. During the electric pulse, charged macromolecules, including DNA, are actively transported by electrophoresis across the cell membrane through these pores (Neumann, et al., Biophys J *71*, 868-77 (1996)). Noncharged molecules can also enter through the pores by passive diffusion. Upon pulse termination, pores reseal over hundreds of milliseconds as measured by recovery of normal membrane conductance values (Ho, 1996, *supra*).

Although electroporation is an established method for implantation of exogenous materials in various cell types including both prokaryotic and eukaryotic cells, e.g., transformation and transfection of cell types ranging from bacteria to mammalian cell lines, its application to brain cells such as neurons and glial cells in intact tissues has been relatively limited. Electroporation has been successful for gene transfer into large numbers of neurons in chick (Atkins, et al., Biotechniques *28*, 94-6, 98, 100 (2000); Sakamoto, et al., FEBS Lett *426*, 337-41 (1998); Koshiba-Takeuchi, et al. Science *287*, 134-7 (2000)) and mouse embryos (Akamatsu, et al., Proc Natl Acad Sci U S A *96*, 9885-90 (1999); Miyasaka, et al., Neuroreport *10*, 2319-23 (1999)). Electroporation is being utilized for neuronal gene transfer since it has many advantages over more common transfection methods including viral gene transfer, gene gun biolistics, lipofection and microinjection. Electroporation lacks the potentially toxic effects of viruses and lipofection and the potential physical damage due to the biolistic gene gun and microinjection. Electroporation is also significantly more efficient than either lipofection, microinjection or gene gun biolistics, in terms of numbers of transfected cells and the intensity of foreign gene expression (See Muramatsu, et al., Biochem Biophys Res Commun *230*, 376-80 (1997)). Few viruses are available which can infect postmitotic brain cells. Viruses are also limited by the size of the foreign DNA that can

be inserted into their genome and thereby transferred to cells. This limits the use of viral gene transfer to study the function of large genes and the use of dicistronic complexes to introduce multiple genes into cells. Finally, lipofection can only be used in proliferating cells and results in transient expression lasting only a few days.

In situations where single-cell gene transfer in intact tissues is desired to express multiple genes, current transfection techniques are insufficient. For many applications, co-expression of multiple genes is desired to allow one to visually identify the transfected cells or to study the interaction of multiple proteins. Co-expression of multiple genes may also be required for assays of brain cell structure following expression of a fluorescent protein along with a gene of interest. Low titre virus can be used to sparsely infect cells, but viral infection is subject to the limitations mentioned above. Although gene-gun biolistics may be utilized to deliver multiple genes, it cannot be used in vivo and is thus limited in its application. Gene transfer by microinjection typically requires that DNA be delivered into the cell nucleus. This method is only applicable to cells plated on a coverslip in which a micropipette can be visualized as it penetrates the nucleus using expensive microscopy equipment. While electroporation is a promising transfection technique, precise targeting is not feasible using traditional, large electrodes. However, one of the powerful attributes of electroporation is the ability to localize transfection by controlling exposure to either DNA or the electric field.

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SUMMARY OF THE INVENTION

An electroporation assembly is provided which includes a container having a distal opening, the container configured to receive a conductive fluid including a substance for delivery into a cell; a first electrode having at least a portion configured to be disposed within the container and in direct electrical communication with the conductive fluid; and a second electrode positioned in proximity to the distal opening for creating an electric field between the electrodes.

Also provided is a method for delivering a substance into a cell which includes providing a container having a distal opening; placing a conductive fluid including a substance in the container; placing the distal opening in proximity to the cell;

and causing an electrical signal to pass through the conductive fluid and the cell wherein the substance passes through the distal opening and enters the cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1a depicts confocal imaging of a single GFP-filled neuron in the tadpole optic tectum. Arrows point to GFP-filled axons.

Figure 1b depicts confocal imaging of a single GFP-filled neuron in a rat hippocampal CA1 pyramidal cell. Arrows point to GFP-filled axons.

Figure 1c depicts confocal imaging of a single GFP-filled neuron in a rat hippocampal CA3 pyramidal cell. Arrows point to GFP-filled axons.

Figure 1d depicts confocal imaging at a higher magnification of the basal dendrites of the CA1 pyramidal cell shown in Figure 1b which shows filling of dendritic spines (arrowheads).

Figure 2a is a pictorial depiction of single-cell electroporation of a neuron *in vivo* by insertion of a glass micropipette filled with a DNA solution into the brain of an anesthetized tadpole. Electrical stimulation delivered between the micropipette and an external ground transfects a single cell at the micropipette tip.

Figure 2b depicts a circuit diagram for delivery of voltage pulses as square pulses or exponential decay pulses. The voltage source is a periodic pulse generator, R_E represents the resistance of the electrode in the tadpole, and R_V represents a variable resistor used to control the time constant (τ) of the decay of the exponential pulse. A switch was used to connect the capacitor to the voltage source or to the electrode and variable resistor.

Figure 2c is a graphical representation which compares the percentage of tadpoles with labeled cells based on the type of voltage pulse. Optimal parameters for single-cell electroporation were dependent on pulse shape and amplitude. All stimuli were delivered 5 times at 1/sec. Exponential decay pulses (τ = 70 ms) produced by the circuit depicted in Figure 2b were more effective than square pulses (1 ms). Highest transfection efficiencies were produced with trains of 1 ms square pulses delivered at 200 Hz.

Figure 3a depicts *in vivo* time-lapse confocal imaging at 2 h intervals 24 h after electroporation and demonstrates typical rapid axonal and dendritic extensions and retractions. Daily imaging up to 6 days following single-cell electroporation demonstrate that neurons grow continuously and appear healthy.

Figure 3b is a graphical representation comparing the number of branches, additions and retractions between neurons containing DiI via iontophoresis and neurons containing GFP via single cell electroporation.

Figure 3c is a graphical representation of growth rate comparing neurons containing DiI via iontophoresis and neurons containing GFP via single cell electroporation. Dendritic growth dynamics at 2 h intervals 24 h after electroporation were similar to the dynamic of tadpole neurons labeled with DiI. The total dendritic arbor growth rates over 24 h of neurons labeled with GFP by single-cell electroporation (SCE) were also similar to neurons labeled with DiI, indicating that electroporation according to the present invention does not adversely affect tectal cell development.

Figure 4a depicts confocal imaging of a tadpole optic tectal neuron cotransfected with pEGFP and pDsRed using single-cell electroporation with GFP fluorescence excited at 488nm to emit green.

Figure 4b depicts confocal imaging of the co-transfected tadpole optic tectal neuron shown in Figure 4a having DsRed fluorescence when excited at 568nm to emit red.

Figure 4c depicts an overlay of confocal imaging of the cotransfected tadpole optical tectal neuron shown in Figures 4a and 4b which appears yellow.

DESCRIPTION OF THE INVENTION

A novel technique is provided herein for insertion of foreign or exogenous substances into individual cells. This technique is particularly well-suited for use on cells within living organisms, e.g., neurons and brain cells including glial cells such as astrocytes, and other cell types, including stem cells, epithelial cell types and muscle cells. In this technique, for example, if the foreign substance is a gene and the target cell is a brain cell, a container having a distal opening, e.g., a glass pipette, containing DNA solution is inserted into the brain of an anesthetized animal with the tip

next to the target cell. Electrical pulses from within the pipette cause DNA to move across the cell membrane into the cell interior. The cell then uses this newly acquired DNA to produce the specific proteins encoded by the DNA. In this manner, the role of cells and particular proteins in brain function can be studied at the single-cell level. In one example of the present invention described in more detail below, a detectable label, e.g., green fluorescent protein was expressed in brain cells of tadpoles and rats, which allowed observation of individual cells growing within the brain over many days.

The present invention is an advancement over current electroporation techniques because it allows exquisite control over the location and number of cells receiving a desired exogenous substance; it can be used at the surface of a single exposed cell or deep within tissue; it can be used in whole live organisms; it is relatively inexpensive and simple; it allows co-transfer of a plurality of materials, e.g., multiple genes, simultaneously; and it lacks many of the toxic side-effects associated with other commonly used gene transfer methods. Most techniques currently available transfer a target substance to a variable number of cells. Single-cell electroporation according to the present invention allows insertion of materials such as genes which provide associated expression of proteins in one cell of a chosen location in an organism while all of the cell's neighbors remain unaffected. Therefore, induced cellular changes are directly due to the foreign DNA introduced in that cell and not to interactions with altered surrounding tissue.

Thus, according to the present invention, molecules to be transferred into a single cell are suspended in a conductive fluid and put into a container having a distal opening such as a syringe, buret or a hollow micropipette, preferably a pulled glass pipette with a sharp tip. In the case of charged molecules such as DNA, the molecule can act as the charge carrier of the conductive solution. The distal opening of the container, preferably having a diameter smaller than the cell diameter, is placed in proximity to and more preferably against the membrane of a target cell. Target cells can be electroporated in culture, in acute tissue preparations, or in the intact organism. Electrical pulses are delivered between an electrode, e.g., a metal wire in the conductive solution in the container and an external ground, such as a metal wire in solution outside of the cell. Electrical pulses induce the temporary formation of pores in the cell membrane. The

molecules of interest can then enter the cell through these pores by passive diffusion, or active electrophoretic motion induced by the electric field if the molecules are charged. The container, cell tissue and/or organism may then be removed. Single cell electroporation according to the present invention utilizes the properties of electroporation commonly used to transfer DNA into large numbers of cells using electric fields between relatively large flat plate electrodes. The use of a container having a distal opening such as a micropipette localizes both exposure to the molecule of interest and the electric field, thereby allowing targeting of electroporation of individual cells.

Electrodes used in accordance with the present invention are conductors which establish electrical contact with a non-metallic portion of a circuit. As such, the electrode is an element that is conductive to electron flow and can be made from conductive materials such as silver, platinum, gold, aluminum, stainless steel, titanium, copper, carbon, alloys of the aforementioned materials and the like. Such materials and alloys are well known in the art. Preferably, the electrodes should be made of a relatively non-toxic material. The shape of the electrode is not deemed to be of critical importance as long as at least a portion thereof is configured to fit in the container having the distal opening. Thus, the electrode may be a simple wire, a strip, a sheet or any other suitable shape.

The container having a distal opening is preferably configured to optimize access to the target cell and, as such, preferably has a streamlined shape at least in the area of the distal opening. The container should be made of an insulating material such as glass or other suitable insulating material known to those skilled in the art. The container is configured to receive at least a portion of the electrode and to hold a conductive fluid containing the substance to be inserted into the target cell. For example, the container can include a second opening (other than the distal opening) which is configured to allow the electrode to enter the interior of the container. As mentioned above, a pulled glass pipette with a sharp tip is particularly preferred. The container may optionally include a support to stabilize the electrode and a removable seal to prevent undesired loss of conductive fluid through the second opening. For example, a one-hole rubber stopper can be utilized to seal the electrode and second opening simultaneously.

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The distance between a first electrode in the container and a second electrode may vary as long as a sufficient electrical field is created to open pores in a target cell membrane. The first electrode in the container should have a higher potential than the second electrode which, as mentioned above, may be a ground. The electrodes should be able to accommodate a wide range of voltages since the field strength may be varied by routine experimentation depending on the inherent resistance of the circuit which is contributed to by the type of cell, the thickness of the tissue undergoing electroporation, the contents of the conductive fluid, the distance between the electrodes and the nature of the substance being implanted into the cell. The distance between the distal opening of the container and the second electrode should be wide enough to accommodate a single cell, tissue sample, or a living organism. It is contemplated that a tissue or cell support made of a relatively conductive material can be interposed between the distal opening and the second electrode. Alternatively, the cell of the sample can be supported by the second electrode itself, e.g., a ground plate. In another embodiment, the tissue sample can be stretched across the locus of the distal opening and in this manner be located between the first and second electrodes.

Electroporation solutions (also referred to herein as conductive fluids) are well-known in the art. Accordingly, those skilled in the art can fabricate or select conductive fluids for use in accordance with the present invention. In certain embodiments, it is contemplated that charged molecules, e.g., DNA, which are intended to be inserted into a target cell may constitute all or a portion of the charged moiety of the conductive fluid. Any suitable substance may be introduced into a target cell according to the present invention including DNA, RNA, proteins, peptides, metals, dyes, pharmaceutical compounds having therapeutic or physiological activity such as drugs, hormones, growth factors, enzymes, vitamins, minerals and the like. Oligonucleotides, chimeric genes, fusion proteins, ligands, receptors, molecular labeling systems such as fluorescent molecules, radiolabels, antibodies, antigens, avidin, streptavidin, biocytin, and biotin are examples of substances which are suitable for electroporation. Examples of fluorescent molecules (fluorochromes), include green fluorescence protein (GFP), color shifted mutants of GFP including red shifted mutants, yellow shifted mutants and blue shifted mutants, amino coumarin acetic acid (AMCA), fluorscein isothiocyanate

(FITC), tetramethylchodamine isothiocyanate (TRITC), Texas Red, Cy3.0, Cy5.0 and dextran conjugates of fluorochromes. Such labels may be used independently or coupled to other molecules such as antibodies, antigens, avidin, streptavidin, and nucleic acid probes. If a particular nucleic acid sequence is the substance to be electroporated into a cell, it may be contained in any suitable vector known in the art. For example, plasmids, cosmids, yeast artificial chromosomes, bacterial artificial chromosomes and the like are all suitable.

Target cells may be electroporated with a variety of electrical pulses. Power sources such as periodic pulse generators are well known in the art and can produce voltage gradients ranging, e.g., from less than about 10 volts/cm to over about 10,000 volts/cm. Square waves can also be generated from pulse generators which produce voltages ranging, e.g., from less than about 10 volts to over about 5,000 volts. Pulse widths may range, e.g., from less than about 0.1 milliseconds (ms) to over about 100 ms. Those with skill in the art can optimize electroporation efficiency for particular substances and target cells by varying the number of electrical pulses delivered, voltage amplitude, pulse shape, and content of the conductive fluid.

Thus, applications for electroporation in accordance with the present invention include: 1) Transfer of DNA or RNA into individual cells for the purpose of controlling protein expression. Both sense sequences for new protein expression, and anti-sense sequences to reduce endogenous protein expression can be introduced; 2) Transfer of DNA into germ cells for the incorporation into chromosomal DNA for the creation of transgenic organisms; 3) Introduction of non-genetic molecules into cells. Both charged and non-charged molecules, including proteins, antibodies, metals, dyes and drugs, can be transferred into individual cells by this technique; and 4) Introduction of DNA and other molecules into stem cells for induction of differentiation, expression of specific proteins, generation of reengineered progenitor cells through insertion of DNA into chromosomes, and to track differentiation and progeny in developmental studies of tissues and organisms. The present invention is well-suited for both *in vitro* and *in vivo* delivery of desired substances into cells.

In one embodiment, the present invention provides the ability to target transfection to individual neurons within the intact central nervous system using single-cell electroporation. Transfection can be focused to individual cells by confining the extent of exposure to DNA and the applied electric field to the tip of a micropipette. Single-cell electroporation according to the present invention has broad application to different cell types as demonstrated by the transfection of individual optic tectal neurons in the Xenopus tadpole brain and pyramidal cells and interneurons in the rat hippocampal slice.

Single-cell electroporation does not appear to have lasting effects on neuronal health. Electroporated neurons expressing GFP had typical morphologies without signs of blebbing or degeneration. Repeated *in vivo* time-lapse imaging of tadpole neurons demonstrated continuous growth for the entire imaging period, up to 6 days following electroporation. See Figure 3(a). In addition, the short-term (2 h) dendritic arbor branch dynamics and 24 h growth rates of neurons following single-cell electroporation were similar to those from cells labeled with the lipophillic dye 0.02% 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI, Molecular Probes) (see Figure 3(c)). This similarity indicates that electroporation, plasmid DNA incorporation, and GFP expression does not interfere with neuronal growth. These findings are supported by a previous study showing that electroporated hippocampal neurons in culture maintain normal synaptic transmission and electrophysiological properties (Teruel, et al., J Neurosci Methods *93*, 37-48 (1999)).

Although, it is likely that optimal stimulation parameters for single-cell electroporation which transfect cells without causing damage is specific to each cell type and tissue preparation, such parameters may be readily determined by routine experimentation by those skilled in the art. Effective stimulation parameters must balance the requirements for temporary pore formation and DNA electrophoresis against the damaging effects of strong electric fields (Neumann, 1996, *supra*). Electric field strengths exceeding optimal parameters may result in too many pores or pores too large to reseal, leading to cell lysis. While relatively brief, high voltage pulses are required to disrupt electrostatic forces maintaining the lipid bilayer structure, longer duration, lower amplitude voltage pulses are required for efficient translocation of DNA across the cell

membrane (Neumann, 1996, *supra*). The efficiency of pulses generated by capacitance discharge which have a high initial peak voltage followed by an exponential decay was therefore tested as described below. Indeed, exponential decay pulses yielded high transfection rates. The highest transfection efficiency, however, was elicited by high frequency trains of short duration square pulses.

Using optimized stimulation parameters, high efficiency of co-electroporating individual neurons with two plasmids was accomplished. In the majority of neurons co-transfected with the genes for GFP and DsRed (a fluorochrome protein mutant similar to GFP), both proteins were detected. However, no cells expressing only DsRed were found and any GFP-only cells were relatively dim. It is possible, therefore, that DsRed may have been present in these cells at levels too low to detect with imaging system utilized, due to the fact that the relative brightness of DsRed is 4 times less than GFP (Matz, et al., comments published erratum appears in Nat Biotechnol 1999 Dec;17(12):1227. Nat Biotechnol *17*, 969-73 (1999)).

Single cell electroporation offers an attractive alternative to other common transfection techniques. One advantage of the present invention is the ease with which multiple genes can be transferred into single cells. When the DNA for different genes is combined and transferred together from the same pipette, the proteins encoded by all the genes are expressed. This feature is a major advance over other electroporation methods such as transferring genes into cells using genetically altered virus, which typically limit the number of genes to one or two. Importantly, electroporation lacks the residual vector agents such as virus, lipofection compounds and gene gun particles which might interfere with neuronal function. In contrast to the apparent long-term health following electroporation, viral infection often leads to cell damage or death within 1 week. Furthermore, the construction of expression vectors such as plasmids for electroporation is relatively routine and inexpensive in contrast to the time and cost required for constructing recombinant viral vectors. Effective electrical stimuli were also produced with relatively inexpensive voltage stimulators common to many neuroscience laboratories.

The ability to target transfection to single cells provides a powerful tool to study gene function in intact nervous systems. The ability to transfer multiple genes into

cells is important to studies of the interaction between different proteins. Transferring genes for colored proteins, such as green fluorescent protein, has proven immensely useful for labeling cells in order to visualize their shapes. These dyes can also be attached to other proteins in order to see where these proteins are located and move within the cell. As demonstrated below, single-cell electroporation according to the present invention was used to fill brain cells with protein dyes in order to observe the cells growth within the intact brain over many days. By introducing other genes along with the gene for green fluorescent protein the effects of various proteins on brain cell growth can be observed. Such observations would not be possible if nearby cells also underwent gene transfer and expressed the dyes because it would be impossible to distinguish the unique shapes of individual cells. GFP expression in individual neurons allows imaging of neuronal morphology without interference of fluorescence from neighboring cells. The ability to restrict transfection to single cells ensures that any induced changes are likely due to a cell-autonomous effects of the exogenous genes, and not secondary influences from transfected neighbors.

The following examples are included to illustrate certain aspects of the present invention and are not intended to limit the invention in any manner whatsoever.

Example I

Preparation of organotypic hippocampal slices

Organotypic slice culture of hippocampus was prepared as described (Stoppini, et al., J Neurosci Methods *37*, 173-82 (1991)). Briefly, hippocampal slices (400 μm) were cut from postnatal 6- to 8-day-old rats with a tissue chopper and incubated on Millicell filters (Millipore) at 37°C, exposed to a combination of 95% O_2 and 5% CO_2 (Musleh, et al., Proc Natl Acad Sci U S A *94*, 9451-6 (1997)). Slices were used for single-cell electroporation after 5 to 7 days in culture. Twenty-four to 48 h after transfection, hippocampal slice cultures were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 15 min, rinsed with 0.1 M PB, and mounted on glass slides with VectaShield (Molecular Probes, Eugene, OR).

Example II

Single-cell electroporation

Transfection of single cells was accomplished using electroporation from DNA-filled micropipettes. Micropipettes with a tip diameter of 2 μm, were pulled on a P-87 Micropipette Puller (Sutter Instrument Company, CA), and filled with solution containing plasmid DNA. Plasmid DNA was purified using Promega Wizard Plus MidiPreps DNA purification system (Promega, Madison, WI) and resuspended at concentrations ranging from 0.25-2.0 μg/μl in dH_2O or 0.1 M phosphate buffer (PB), pH 7.4. A silver wire was placed inside the micropipette in contact with the internal DNA solution. Micropipette holders were mounted on course, 3-axis, manual micromanipulators. Voltage pulses generated by a Grass SD9 Stimulator (Grass-Telefactor, West Warwick, RI) were delivered between the micropipette electrode (anode) and a silver wire ground electrode (cathode) placed under the tadpole or brain slice.

For single-cell electroporation in the intact tadpole brain, stage 46-48 tadpoles were anesthetized with 0.02% 3-aminobenzoic acid ethyl ester (MS222, Sigma) in Steinberg's solution (pH 7.4). Anesthetized tadpoles were placed on top of a moistened Kimwipe on the stage of an Olympus BX50 microscope equipped with a 20X long working distance lens. The DNA-containing micropipette was inserted blind into the optic tectum of the tadpole brain in a region with a high density of cell bodies. The resistance of the micropipette electrode ranged from 15 to 30 MΩ in the tadpole brain.

For transfection of single cells in hippocampal slice cultures, a Millicell culture well containing slices was placed in a 35 mm petri dish containing culture medium, on the stage of a dissecting microscope. The micropipette containing DNA was inserted into the stratum pyramidale of areas CA1 or CA3 within the hippocampal slice. Micropipette resistance in the hippocampal slice ranged from 10 to 15 MΩ.

Stimulation parameters for single-cell electroporation including voltage pulse amplitude, duration, and shape were tested for transfection efficiency in the tadpole brain. Transfection efficiency was measured by expression of fluorescence following electroporation with the plasmid pEGFP (Clontech Laboratories, Palo Alto, CA), a plasmid carrying the gene for enhanced EGFP driven by a CMV promoter. Two voltage

pulse shapes were compared, square pulses and capacitance discharge pulses with a high initial peak followed by an exponential decay. Square pulses were generated with the Grass SD9 stimulator and were delivered as individual stimuli or 1 s trains of 1 ms square pulses. Trains of 1 ms square pulses were delivered at 50 or 200 Hz for 1 sec. To form exponential decay pulses, voltage pulses from the Grass stimulator (200 Hz, 4 ms) were used to charge a capacitor (0.4 - 3 μF) which was then allowed to discharge across the electrode. The time constant (τ) of decay of the capacitance discharge was controlled by a variable resistor in parallel with the capacitor. Time constants tested ranged from 20 to 70 ms. Voltages for both square and exponential decay pulses were varied between 10 and 70 V, and in each case single stimuli were compared with 5 stimuli delivered at 1 Hz. Limited clogging allowed repeated use of the same micropipette at different sites. Four sites were electroporated in each tadpole. The optimal stimulation parameters for single-cell transfection in the tadpole brain were then tested in the hippocampal slice culture. Tadpoles and brain slices were examined for GFP expression 24 to 48 h after electroporation using a Nikon Optiphot microscope with epifluorescence (20X objective, NA 0.75).

Electroporation of DNA from a micropipette resulted in transfection of single neurons in the Xenopus tadpole brain and the cultured rat hippocampal slice (Figures 1a-1d). In cells transfected with pEGFP, bright GFP fluorescence in somata, dendrites and axons could be detected within 12 h. In hippocampal pyramidal cells, GFP completely filled all processes including dendritic spines. Fluorescence increased over 2 to 3 days, even with repeated imaging (Figures 3a-3c), and remained high for more than 2 weeks.

Single-cell electroporation efficiency tested in the tadpole brain was dependent on stimulation parameters including the number of pulses delivered, voltage amplitude and pulse shape (Figures 2b and 2c). Three types of stimuli were tested – square pulses, exponential decay pulses produced by capacitance discharge, and high frequency trains of square pulses. Delivery of 5 repeated individual pulses or trains (1 sec in duration) with 1 sec interstimuli intervals were much more effective than individual stimuli, and therefore, 5 repeated pulses or trains were delivered for each parameter tested. Initial tests with 1 ms square pulses at voltages ranging from 10 to 50

V produced low numbers of single cell transfections (Figure 2c). Transfection efficiency was greatly enhanced by exponential decay pulses with $\tau = 70$ ms. Transfection efficiency using exponential decay pulses were voltage dependent with highest rates at a voltage peak of 20 V. Trains of 1 ms square pulses yielded high transfection rates and were frequency and voltage dependent. The highest transfection rates in tadpole brains were with 200 Hz trains of 1 ms square pulses at 50 V. In the rat hippocampal slice culture, trains of 200 Hz stimuli (1 ms pulses) at 20 V were the most effective for transfection of individual neurons, including CA1 and CA3 pyramidal cells (Figures 1b-1d), and CA1 interneurons. Transfection efficiency was not noticeably affected by DNA concentration in the range of 0.25 to 2.0 μg/μl, the inclusion of $CaCl_2$ in the DNA solution, or resuspension of DNA in dH_2O or phosphate buffer (PB).

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Example III

Neuronal growth and dendritic branch dynamics

Time-lapse confocal imaging was used to determine whether single-cell electroporation and subsequent exogenous protein expression interferes with normal neuronal morphology and growth. Both short-term (2 h) dendritic branch dynamics and total dendritic arbor growth over 24 h were compared between neurons transfected with pEGFP by single-cell electroporation and single neurons labeled with DiI (Molecular Probes), a dye commonly used for imaging neuronal morphology (Wu, G. Y. and Cline, H. T., Science *279*, 222-6 (1998)). Eleven optic tectal neurons were transfected with pEGFP (0.5 μg/μl) using the optimal stimulation parameters (200 Hz trains, 50 V). Fifteen neurons in the optic tectum were labeled by iontophoresis of DiI dissolved in ethanol, using 3-10 pulses of 200 ms positive current pulses (1-10 nA). Starting 24 h after electroporation, cells were imaged 5 times at 2 h intervals, and then imaged the following day. In addition, some cells were subsequently imaged once-daily for up to 6 days. Dendritic arbors were reconstructed by tracing the portion of the neuron in each acquired optical section onto an acetate sheet until the entire neuron was drawn. Total dendritic branch length was measured from scanned drawings of cells using the program NIH Image 1.61. To analyze the arbor dynamics, drawings of cells from sequential timepoints were superimposed to identify added and retracted branches.

Repeated confocal imaging of neurons in anesthetized tadpoles was utilized to monitor neuronal morphology, dendritic arbor branch dynamics at 2 h intervals, and dendritic arbor growth rates over several days (Figures 3a-3c). These parameters were compared in GFP-expressing cells and cells labeled with the DiI. In both DiI-labeled cells and neurons electroporated with GFP, dendritic arbors grew continuously, without signs of dendritic or axonal swelling or degeneration. Dendritic arbor branch additions and retractions measured at 2 h intervals were comparable in the two groups (additions: GFP = 55.1 ± 7.6 branches/2 h; DiI = 52.2 ± 6.5 branches/2 h; retractions: GFP = 48.6 ± 7.2 branches/2 h; DiI = 39.8 ± 4.5 branches/2 h, expressed as mean ± SE). Growth rate over 24 h, measured as the total dendritic branch length on day 2 (time after labeling) subtracted by the branch length on day 1, was also similar between groups (GFP = 193.7 ± 21.7 μm/24 h; DiI = 229.5 ± 38.1 μm/24 h).

Example IV

Single cell electroporation of Glial Cells

For single-cell electroporation in the rat brain in vivo, rats 14-20 days old were anesthetized with a combination of xylazine (0.03 mg/g body weight) and ketamine (0.56 mg/g body weight), and placed in a stereotaxic apparatus. Body temperature was maintained with a heating pad placed under the rat. A small incision was made in the skin and a small hole (~4mm) was opened in the skull exposing the brain. A ground electrode was attached to the cut skin using an alligator clip. A micropipette filled with a solution containing plasmid DNA carrying the gene for EGFP (0.5 μg/μl) was inserted into the brain tissue at multiple site depths ranging from 100 to 400 μm. Brief electrical stimuli (1 s trains of 1 ms square pulses at 200 Hz, 20 to 50 V) were delivered between a silver wire within the micropipette and the ground electrode. Following electroporation, the incision was closed and sealed with surgical glue. Two to 4 days following electroporation, rats were euthanized and purfused with phosphate buffered saline (50 ml) followed by 4% paraformaldehyde (50 ml) to fix the brain. Following perfusion, brains were removed and submerged in 4 % paraformaldehyde for 12 to 24 hours at 4^0C. Brains were then cut into 100 or 200 μm thick sections with a vibratome which were then mounted on subbed glass slides with VectaShield mounting solution (Molecular Probes)

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and covered with glass coverslips. Tissue sections were screened for GFP-expressing cells using epifluorescence and transfected cells were imaged with confocal microscopy. Micropipette electroporation in the intact rat brain was shown to have resulted in GFP expression in astrocytes at the site of stimulation.

Example V

Electroporation of multiple plasmids

In order to determine the efficiency of co-transfecting multiple independent genes, the plasmids pEGFP and pDsRed (Clontech Laboratories) were used together for single-cell electroporation. Fifty neurons in the tadpole optic tectum were transfected using micropipettes containing a mixture of both plasmids (0.5 μg/μl of each). Starting 12 h after electroporation and for up to 4 days after, anesthetized tadpoles were examined for GFP and DsRed fluorescence by confocal microscopy.

Single-cell electroporation with two independent plasmids encoding GFP and DsRed resulted in co-expression of both fluorescent proteins in 92% (46 out of 50) of cells (Figures 4a-4c). While GFP fluorescence could be detected within 12 h and steadily increased in intensity over 3 days, detection of DsRed expression was relatively delayed, initially detectable after 24 to 36 h, and increasing over 4 to 5 days. Bright GFP and DsRed fluorescence persisted for more than 2 weeks, the longest time examined. Following co-electroporation, 46 out of 50 cells expressed detectable levels of both GFP and DsRed. The remaining 4 cells exhibited dim GFP with no detectable DsRed. In all cases, GFP was brighter than DsRed and DsRed was never detected without GFP.

Example VI

Confocal imaging

GFP and DsRed fluorescence following single-cell electroporation, and DiI labeling was imaged with a confocal microscope comprised of a krypton-argon laser and a Noran XL laser scanning confocal attachment mounted on an upright Nikon Optiphot microscope, using a 40x Nikon oil immersion lens (1.30 NA). Tadpoles were anesthetized with 0.02% MS222 prior to imaging and were allowed to recover between imaging sessions. Hippocampal slice cultures were fixed and mounted on slides before

imaging. Images were collected through the entire extent of the neuron at steps of 1 or 2 μm in the z-dimension. Eight to 16 frames were averaged for each optical section. GFP was excited at 488 nm and emitted light was filtered between 500 nm and 550 nm. DsRed was excited at 568 nm and emitted light between 578-632 nm was detected. DiI was excited with 568 nm and emitted light with greater than 590nm was detected.

It will be understood that various modifications may be made to the embodiments and aspects disclosed herein. For example, it is contemplated that circuits having topology other than that depicted in Figure 2(b) such as series RC or series RL circuits can be utilized in accordance with the present invention. Therefore, the above description should not be viewed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the spirit and scope of the claims appended hereto.

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WHAT IS CLAIMED IS:

1. An electroporation assembly comprising:

a container having a distal opening, the container configured to receive a conductive fluid including a substance;

a first electrode having at least a portion configured to be disposed within the container and in direct electrical communication with the conductive fluid; and

a second electrode positioned in proximity to the distal opening for creating an electric field between the electrodes.

2. An electroporation assembly according to claim 1 wherein the container is selected from the group consisting of pipette, buret and syringe.

3. An electroporation assembly according to claim 2 wherein the pipette is a micropipette.

4. An electroporation assembly according to claim 3 wherein the micropipette is a glass pulled pipette having a sharp tip opening having a diameter less than the diameter of a target cell.

5. An electroporation assembly according to claim 1 further comprising a tissue support for maintaining a cell, tissue or an organism between the distal opening and the second electrode.

6. An electroporation assembly according to claim 1 further comprising a power supply.

7. An electroporation assembly according to claim 6 wherein the power supply is a periodic pulse generator.

8. An electroporation assembly according to claim 1 wherein the first and second electrodes are independently made of a conductive material selected from the group consisting of silver, copper, stainless steel, aluminum, platinum, gold, carbon and alloys thereof.

9. A method for delivering a substance into a cell comprising:
providing a container having a distal opening;
placing a conductive fluid including a substance in the container;
placing the distal opening in proximity to the cell; and
causing an electrical signal to pass through the conductive fluid and the cell wherein the substance passes through the distal opening and enters the cell.

10. A method for delivering a substance into a cell according to claim 9 wherein the container is selected from the group consisting of pipette, buret and syringe.

11. A method for delivering a substance into a cell according to claim 10 wherein the pipette is a micropipette.

12. A method for delivering a substance into a cell according to claim 11 wherein the micropipette is a glass pulled pipette having a sharp tip opening having a diameter less than the diameter of the cell.

13. A method for delivering a substance into a cell according to claim 9 wherein the electrical signal passes between first and second electrodes, the first electrode having at least a portion thereof disposed within the container and in direct electrical communication with the conductive fluid.

14. A method for delivering a substance into a cell according to claim 9 wherein the cell is in direct contact with the distal opening.

15. A method for delivering a substance into a cell according to claim 9 wherein the substance is selected from the group consisting of nucleic acid, dye, protein, antibody, antigen, peptide, metal, pharmaceutical compound, a radiolabeled derivative of the foregoing and combinations thereof.

16. A method for delivering a substance into a cell according to claim 15 wherein the nucleic acid is contained in a vector.

17. A method for delivering a substance into a cell according to claim 15 wherein the dye is a fluorochrome.

18. A method for delivering a substance into a cell according to claim 15 wherein the protein is a fluorochrome.

19. A method for delivering a substance into a cell according to claim 18 wherein the protein is green fluorescent protein or a red shifted mutant thereof.

20. A method for delivering a substance into a cell according to claim 15 wherein the nucleic acid is a nucleic acid encoding a fluorescent protein.

21. A method for delivering a substance into a cell according to claim 20 wherein the fluorescent protein is green fluorescent protein or a color shifted mutant thereof.

22. A method for delivering a substance into a cell according to claim 9 wherein the electrical signal is generated by a periodic pulse generator.

23. A method for delivering a substance into a cell according to claim 22 wherein the electrical signal is a square pulse.

24. A method for delivering a substance into a cell according to claim 23 wherein the electrical signal is a high frequency train of square pulses.

25. A method for delivering a substance into a cell according to claim 22 wherein the electrical signal is an exponential decay pulse.

26. A method for delivering a substance into a cell according to claim 9 wherein the cell is a neuron.

27. A method for delivering a substance into a cell according to claim 9 wherein the cell is a brain cell selected from the group consisting of neuron and glial cell.

28. A method for delivering a substance into a cell according to claim 9 wherein the method is conducted *in vivo*.

29. A method for delivering a substance into a cell according to claim 9 wherein the method is conducted *in vitro*.

ABSTRACT

A single cell electroporation assembly and method involve delivery of a substance into a single cell. The substance is combined into an electroporation fluid and placed into a container having a distal opening. The distal opening of the container is placed in proximity to a target cell. Electrical pulses are delivered between a first electrode which is at least partially disposed in the container, and a second electrode outside the cell, the cell being positioned between the distal opening and the second electrode. The electrical pulses induce temporary formation of pores in the cell membrane and the substance enters the cell through the pores by passive diffusion or by active electrophoretic motion.

Figure 1

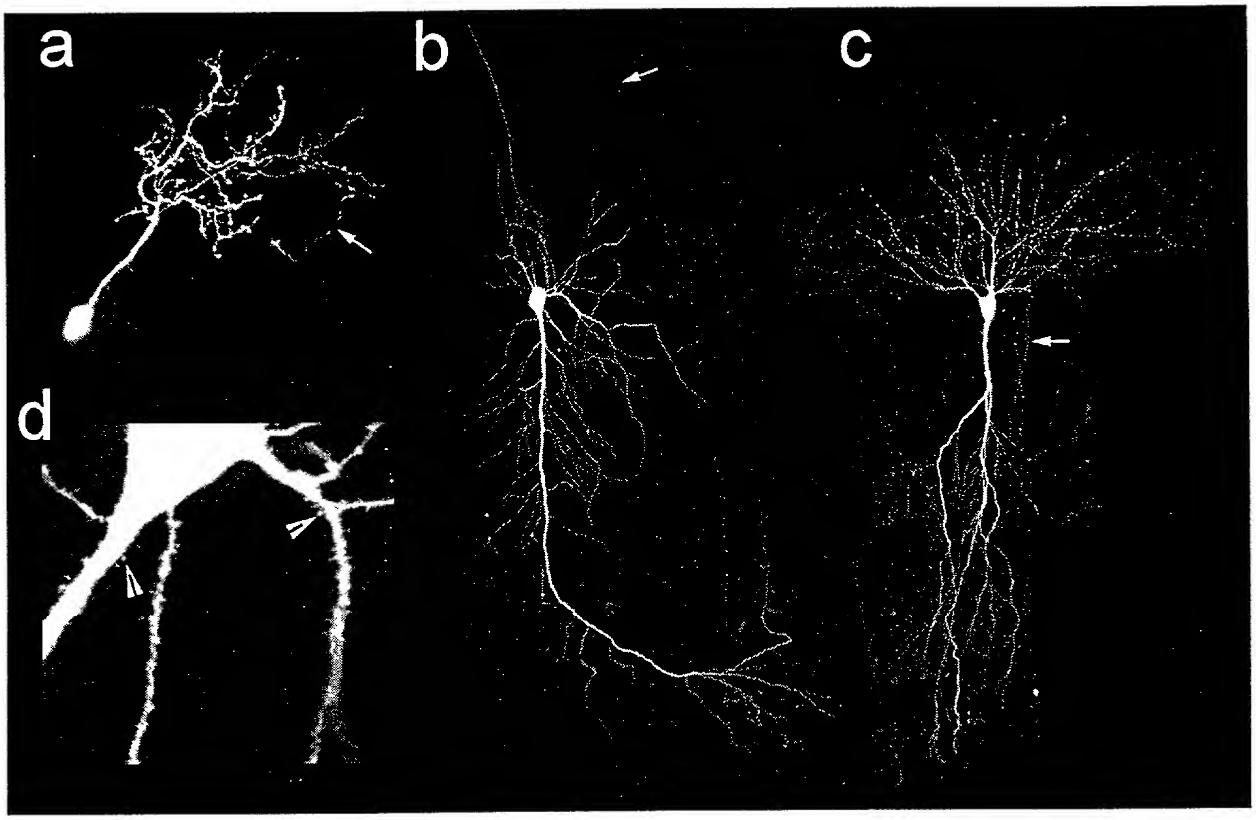
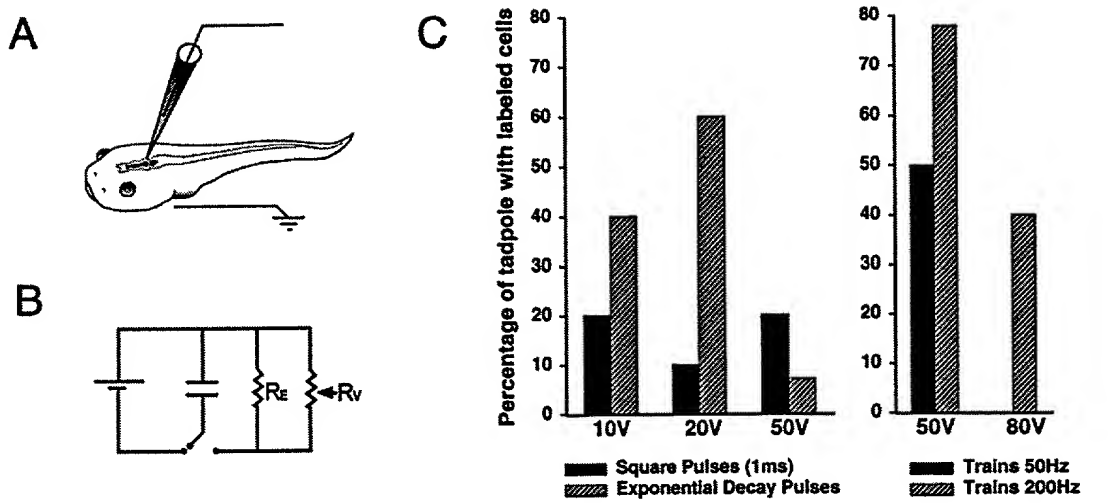
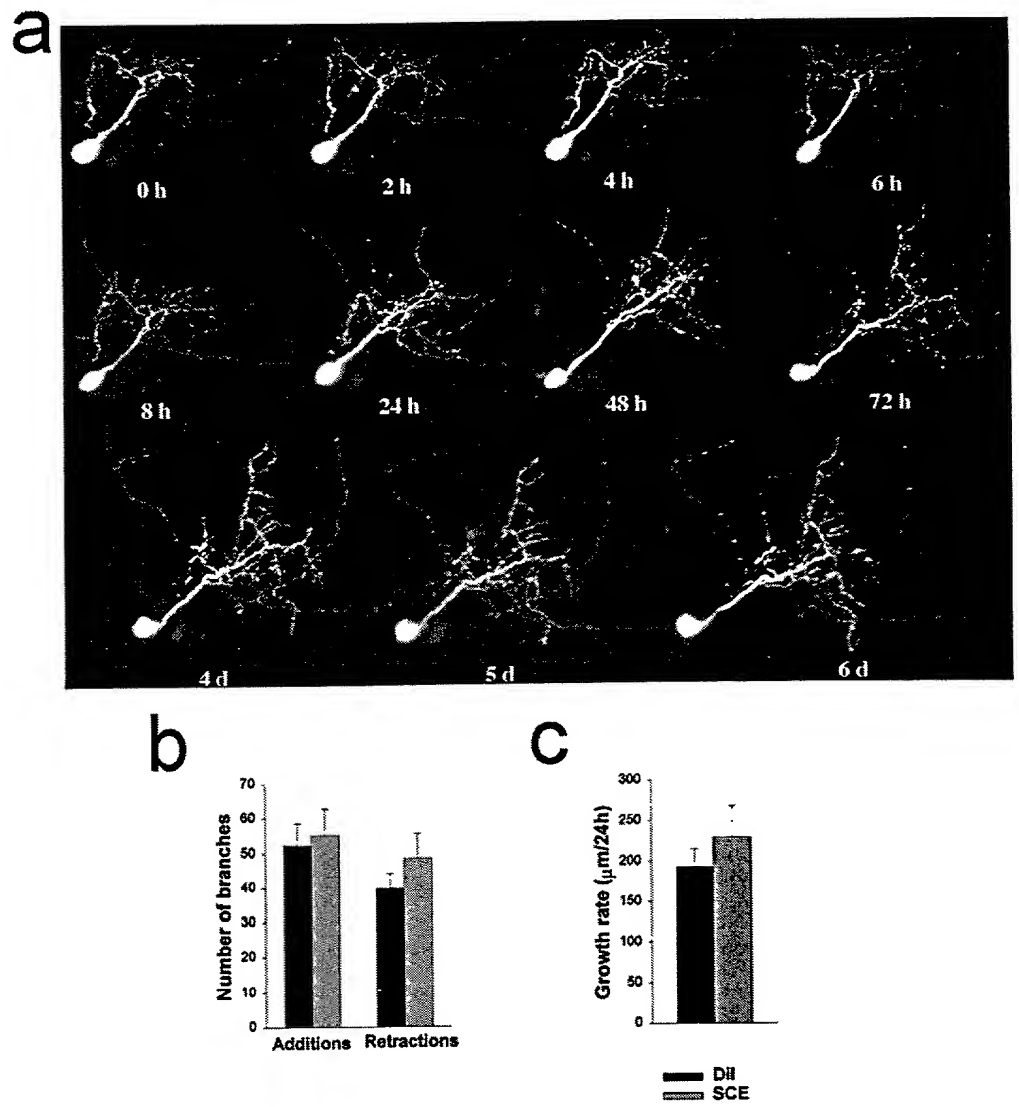


Figure 2



DOCKET ALARM

Figure 3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2 h
4 h
6 h
8 h
24 h
48 h
72 h
4 d
5 d
6 d
b
Number of branches
70
60
50
40
30
20
10
0
Additions
Retractions
c
Growth rate (μm/24h)
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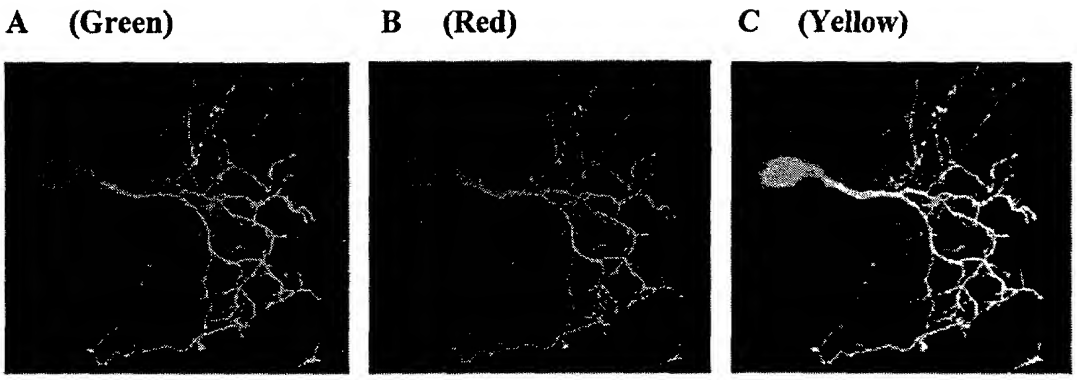
250
200
150
100
50
0
DII
SCE

Figure 4



PATENT

Attorney's Docket No. 1079-2

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION OR CIP)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type: *(check one applicable item below)*

- ■ original
- ☐ design
- ☐ supplemental

NOTE: If the declaration is for an International Application being filed as a divisional, continuation or continuation-in-part application do not check next item; check appropriate one of last three items.

- ☐ national stage of PCT

NOTE: If one of the following 3 items apply then complete and also attach ADDED PAGES FOR DIVISIONAL, CONTINUATION OR CIP.

- ☐ divisional
- ☐ continuation
- ☐ continuation-in-part (CIP)

INVENTORSHIP IDENTIFICATION

WARNING: If the inventors are each not the inventors of all the claims an explanation of the facts, including the ownership of all the claims at the time the last claimed invention was made, should be submitted.

My residence, post office address and citizenship are as stated below next to my name, I believe I am the original, first and sole inventor *(if only one name is listed below)* or an original, first and joint inventor *(if plural names are listed below)* of the subject matter which is claimed and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

SINGLE CELL ELECTROPORATION

SPECIFICATION IDENTIFICATION

the specification of which: *(complete (a), (b) or (c))*

(a) ■ is attached hereto.

(b) ☐ was filed on _____ as ☐ Serial No. 0 /_____ or ☐ Express Mail No., as Serial No. not yet known _____ and was amended on _____ *(if applicable)*.

NOTE: Amendments filed after the original papers are deposited with the PTO which contain new matter are not accorded a filing date by being referred to in the declaration. Accordingly, the amendments involved are those filed with the application papers or, in the case of a supplemental declaration, are those amendments claiming matter not encompassed in the original statement of invention or claims. See 37 C.F.R. 1.67.

(c) ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____ *(if any)*.

ACKNOWLEDGMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.

☐ and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, and

☐ in compliance with this duty there is attached an information disclosure statement in accordance with 37 C.F.R. 1.98.

PRIORITY CLAIM (35 U.S.C. §119)(a)-(d)

I hereby claim foreign priority benefits under Title 35, United States Code, §119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) ■ no such applications have been filed.
(e) ☐ such applications have been filed as follows.

NOTE: Where item (c) is entered above and the International Application which designated the U.S. itself claimed priority check item (e), enter the details below and make the priority claim.

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. §119(a)-(d)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING *(day, month, year)*	PRIORITY CLAIMED UNDER 35 U.S.C. 119
			☐ YES ☐ NO
			☐ YES ☐ NO

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S) (35 U.S.C. §119(e))

I hereby claim the benefit under Title 35, United States Code, §119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	FILING DATE
__/______________	__________
__/______________	__________

ALL FOREIGN APPLICATION(S), IF ANY FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

____

____

____

NOTE: If the application filed more than 12 months from the filing date of this application is a PCT filing forming the basis for this application entering the United States as (1) the national stage, or (2) a continuation, divisional, or continuation-in-part, then also complete ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR CIP APPLICATION for benefit of the prior U.S. or PCT application(s) under 35 U.S.C. §120.

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POWER OF ATTORNEY

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(List name and registration number)*

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DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

NOTE: Carefully indicate the family (or last) name as it should appear on the filing receipt and all other documents.

Full name of **sole or first joint inventor** Kurt Haas
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ADDED PAGE(S) WHICH FORM A PART OF THIS DECLARATION

☐ Signature for subsequent joint inventors.

Number of pages added ____.

☐ Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor.

Number of pages added ____.

☐ Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 C.F.R. §1.47.

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☐ Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (CIP) application.

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☐ Authorization of attorney(s) to accept and follow instructions from representative.

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